

Role of IGF Signaling in Olfactory Sensory Map Formation and Axon Guidance

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DOI 10.1016/j.neuron.2008.01.027

SUMMARY

Olfactory neurons project their axons to spatially invariant glomeruli in the olfactory bulb, forming an ordered pattern of innervation comprising the olfactory sensory map. A mirror symmetry exists within this map, such that neurons expressing a given receptor typically project to one glomerulus on the medial face and one glomerulus on the lateral face of the bulb. The mechanisms underlying an olfactory neuron's choice to project medially versus laterally remain largely unknown, however. Here we demonstrate that insulin-like growth factor (IGF) signaling is required for sensory innervation of the lateral olfactory bulb. Mutations that eliminate IGF signaling cause axons destined for targets in the lateral bulb to shift to ectopic sites on the ventral-medial surface. Using primary cultures of olfactory and cerebellar neurons, we further show that IGF is a chemoattractant for axon growth cones. Together these observations reveal a role of IGF signaling in sensory map formation and axon guidance.

INTRODUCTION

In sensory systems, ordered patterns of neuronal connections represent information about complex stimuli from the external world. In the visual system, for example, retinal ganglion cells form a point-to-point topographic map in their projection to thalamic or midbrain structures, representing information about the position of the stimulus in visual space (McLaughlin and O'Leary, 2005). Similarly, sensory neurons in the somatosensory and auditory systems maintain neighbor relationships when projecting to the thalamus, resulting in a spatial map of the body surface or sound frequency, respectively (Killackey et al., 1995; Rubel and Fritzsch, 2002). In contrast, the projection of primary olfactory sensory neurons to their first relay in the brain comprises a

discontinuous map; neurons expressing a given odorant receptor, while distributed broadly in the peripheral sensory epithelium (Ressler et al., 1993; Vassar et al., 1993), converge to discrete and spatially invariant glomeruli in the olfactory bulb (Mombaerts et al., 1996; Mori et al., 2006; Ressler et al., 1994; Vassar et al., 1994). The pattern of these convergent connections forms the anatomical basis for the olfactory sensory map. This sensory map displays a mirror symmetry, such that odorant receptor-specific neurons typically extend their axons to one glomerulus in the medial hemisphere and another glomerulus in the lateral hemisphere of the olfactory bulb (Mombaerts et al., 1996; Nagao et al., 2000; Ressler et al., 1994; Vassar et al., 1994).

How is the olfactory sensory map established during development? Olfactory neurons express just one odorant receptor allele from a repertoire of ~1000 odorant receptor genes (Chess et al., 1994). The selection of an odorant receptor gene not only determines the specificity of the cell for odorants, but also influences the targeting of its axon in the olfactory bulb (Feinstein and Mombaerts, 2004; Imai et al., 2006; Mombaerts et al., 1996; Wang et al., 1998). While the odorant receptor plays a critical role in determining the projection pattern of olfactory sensory axons, it does not appear to be the sole determinant of glomerular position (Imai et al., 2006; Mombaerts et al., 1996; Wang et al., 1998). Thus, the formation of the olfactory sensory map is thought to involve a hierarchy of cues and axon guidance decisions, which together function to direct the axons of neurons expressing the same odorant receptor to common targets in the olfactory bulb (Lin and Ngai, 1999; St John et al., 2002). However, numerous studies addressing the role of classical axon guidance cues and cell adhesion molecules in the developing olfactory system have yet to provide a complete understanding of how the stereotyped glomerular map is formed with such precision (Cho et al., 2007; Cutforth et al., 2003; Imai et al., 2006; Montag-Sallaz et al., 2002; Puche et al., 1996; Schwarting et al., 2000; Serizawa et al., 2006; Tisay et al., 2000; Treloar et al., 1997; Walz et al., 2002, 2006) (reviewed in Imai and Sakano, 2007; Mombaerts, 2006).

In the present study, we examine the role of insulin-like growth factor (IGF) signaling in axon guidance using the developing

olfactory system as our primary model. The IGF family of signaling effectors includes two secreted polypeptide ligands, IGF1 and IGF2, which bind to and activate a common receptor tyrosine kinase, the type 1 IGF receptor (IGF1R) (Efstratiadis, 1998). Developmentally, IGF signaling plays an important role in determining body size by promoting cell proliferation and survival (Efstratiadis, 1998); null mutations in *Igf1*, *Igf2*, or *Igf1r* in mice result in severe growth deficiency phenotypes (Baker et al., 1993; DeChiara et al., 1990; Liu et al., 1993). In the nervous system, IGF signaling apparently mediates neuronal survival and proliferation (Beck et al., 1995; Chrysis et al., 2001; Ye et al., 1996). In addition, IGF signaling has recently been shown to regulate growth cone expansion (Laurino et al., 2005), promote axon outgrowth in corticospinal neurons (Ozdinler and Macklis, 2006), and play a role in establishing neuronal polarity in cultured hippocampal neurons (Sosa et al., 2006). Thus, a role for IGF signaling in neuronal patterning is beginning to emerge that is distinct from the classical view of IGFs as growth-promoting factors. It remains unclear, however, whether IGFs play a wider role in neuronal patterning or serve as instructive cues affecting axon guidance and axon targeting decisions.

Here we describe results indicating that both IGF1 and IGF2 are expressed by cells in and surrounding the olfactory bulb, whereas IGF1R is expressed in a complementary fashion on olfactory sensory axons. Using a series of IGF mutant mice, we show that the loss of IGF signaling causes a severe misrouting of olfactory sensory axons away from the lateral olfactory bulb to ectopic ventral-medial positions, resulting in a dramatic perturbation of the mirror-symmetric olfactory sensory map. We further demonstrate that IGF1 can act as a chemoattractant for growth cones of cultured olfactory neurons as well as cultured cerebellar granule neurons. These data demonstrate the importance of IGF signaling in the formation of the olfactory sensory map, and suggest a general role of IGFs as instructive axon guidance cues.

RESULTS

IGF Ligands and Their Receptor Are Expressed in Complementary Patterns in the Developing Olfactory System

We previously performed a microarray-based screen to identify candidate genes expressed in the olfactory bulb that could act to guide olfactory axons to their target glomeruli (Lin et al., 2004). One candidate signaling pathway identified by this screen was the IGF signaling pathway. To characterize further the IGF signaling components in the olfactory system, we performed immunohistochemistry on the developing olfactory system at embryonic (E) day 14.5 and 18.5 for IGF1, IGF2, and IGF1R. At E14.5, IGF1 is expressed circumferentially in the developing bulb, just within and in close apposition to the developing olfactory nerve layer (Figure 1A). Interestingly, at rostral positions there appears to be a gradient of IGF1 along the medial-lateral axis, such that expression is higher in the lateral portion of the olfactory bulb. This gradient of expression diminishes and eventually reverses to display a medial > lateral bias more caudally. Localization of olfactory marker protein (OMP), a marker for all olfactory neurons and their axons, reveals a corresponding pattern of olfactory sensory innervation at this stage of development.

The medial-lateral gradient of IGF1 expression is not readily detectable at E15.5 (data not shown), and by E18.5 IGF1 localizes to the mitral cell and glomerular layers (which are more distinct at this stage) throughout the olfactory bulb (Figure 1B).

Consistent with previous RNA in situ hybridization results (Lin et al., 2004; Stylianopoulou et al., 1988), IGF2 protein is expressed in the leptomeninges surrounding the bulb. The highest expression is in the dorsal region (seen as diffuse staining at E14.5, becoming more discrete by E18.5), whereas expression is diminished in the rostral-ventral region (Figures 1A and 1B). Cells of the cribriform plate also express IGF2 (bracket in Figure 1B), where it may interact with olfactory sensory axons as they traverse this structure en route to the olfactory bulb. Together these observations confirm that the two known IGF ligands are expressed in locations in which they could interact with olfactory sensory axons entering the olfactory bulb.

In order for olfactory neurons to respond to olfactory bulb-derived IGF signals, these cells must express IGF1R, the receptor mediating both IGF1 and IGF2 signaling. Similar to previous observations in the rat olfactory epithelium (Ferrari et al., 2003; Suzuki and Takeda, 2002), at E14.5 we found IGF1R to be expressed in immature and mature olfactory neurons, as well as in the underlying sensory axon fascicles (Figure S1 available online), showing no obvious restrictions along the three principle axes (medial-lateral, dorsal-ventral, or rostral-caudal) of the olfactory epithelium (Figure 1A). IGF1R is also present within the olfactory bulb nerve layer at E14.5 (data not shown), and IGF1R-positive axons can be observed entering the glomerular layer by E18.5 (Figure 1B). Thus, the olfactory sensory axons express IGF1R, which presumably makes them able to respond to IGF cues found in the olfactory bulb as they grow into and innervate this structure.

IGF Signaling Is Required for the Normal Projection of Olfactory Sensory Axons in the Olfactory Bulb

The complementary expression of IGF ligands in the olfactory bulb and IGF1R in olfactory sensory axons suggests a role for IGF signaling in the projection of primary sensory axons in the olfactory bulb. We tested this hypothesis by evaluating the projection patterns of olfactory sensory neurons in mice harboring a targeted deletion in the *Igf1r* gene (Liu et al., 1993). To facilitate the visualization of olfactory sensory axons, we examined mice expressing a tau:lacZ fusion protein in all olfactory neurons under the control of the OMP promoter (Mombaerts et al., 1996). The innervation of the olfactory bulb by olfactory neurons was visualized by immunohistochemical staining using an anti- β -galactosidase antibody. Because homozygous *Igf1r*^{-/-} mutants die immediately after birth (Liu et al., 1993), the olfactory projection pattern was assessed at embryonic stages, when some, but not all, glomeruli have formed. Staining for β -galactosidase in coronal tissue sections from embryos as early as E15.5 demonstrates a striking defect in *Igf1r*^{-/-} mice compared with control *Igf1r*^{+/+} or *Igf1r*^{+/-} littermates. Whereas olfactory axons in control mice project to and innervate the entire circumference of the olfactory bulb, homozygous mutant mice show a marked decrease in innervation of the lateral face of each bulb (compare Figure 2A and 2B). Similar results were observed at E18.5 (compare Figure 2C and 2D), as well as at E16.5 and E17.5 (data not

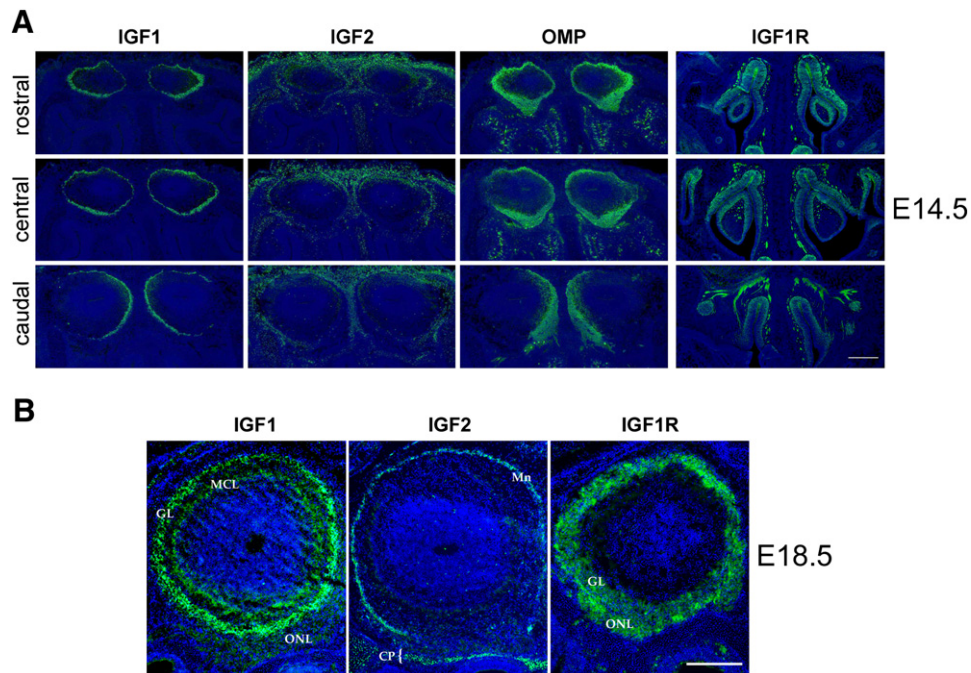


Figure 1. IGF Signaling Components Are Expressed in the Developing Olfactory System

(A) Representative coronal tissue sections from the rostral, center, and caudal regions of the olfactory bulb and epithelium of E14.5 mice were labeled with IGF1, IGF2, IGF1R, or olfactory marker protein (OMP) antibodies. IGF1, IGF2, and OMP immunoreactivity (green) is shown from representative adjacent tissue sections taken at each position along the rostral-caudal axis. At this stage, IGF1 localizes in each bulb immediately inside the olfactory nerve layer (likely in the developing mitral cell and glomerular layers), showing a slight lateral > medial bias in expression rostrally and a pronounced medial > lateral bias caudally. IGF2 immunoreactivity is observed both in the overlying skin and in the leptomeninges of the olfactory bulb, with an exclusion from the ventral bulb. OMP expression—reflecting the disposition of the olfactory sensory axons in the olfactory nerve layer—reveals innervation roughly approximating the location of IGF1 in underlying bulb structures. In the olfactory epithelium, IGF1R is localized to immature and mature olfactory neurons throughout the olfactory sensory epithelium, and to the underlying olfactory axon fascicles (see Figure S1).

(B) Coronal tissue sections of olfactory bulbs from E18.5 animals were analyzed by immunohistochemistry for IGF1, IGF2, and IGF1R expression. IGF1 is expressed in the mitral cell layer (MCL) and glomerular layer (GL) of the olfactory bulb. IGF2 is found in the leptomeninges (Mn) surrounding the bulb as well as in the bone of the cribriform plate (CP and bracket). Note that the expression of IGF2 in the leptomeninges does not completely surround the bulb in this tissue section. IGF1R localizes to the olfactory neuron axons within the olfactory nerve layer (ONL) and within the glomerular layer (GL). Nuclei of cells were visualized with Hoechst 33342 (blue). Dorsal is to the top in all panels; medial is to the right in (B).

Scale bar, 200 μ m in (A) and 100 μ m in (B).

shown). Analysis of serial tissue sections collected along the rostral-caudal axis reveals the paucity of lateral innervation throughout the olfactory bulb in *Igf1r*^{-/-} mice, although this effect is somewhat less pronounced at rostral-most positions (Figure S2). By contrast, in wild-type animals the entire circumference of the olfactory bulb is innervated, with a slight bias toward the lateral face in the rostral bulb (Figure S2).

The appearance of the observed innervation defect relatively early in the development of the olfactory projection (E15.5) suggests that IGF signaling is required for the initial innervation of the lateral olfactory bulb. The phenotype appeared to be fully penetrant and was observed in 2/2 mutants at E15.5 (compared with 3 heterozygous control littermates), 2/2 mutants at E16.5 (2 heterozygous controls), 1/1 mutant at E17.5 (2 heterozygous controls), and 13/13 mutants at E18.5 (6 wild-type or heterozygous controls). In *Igf1r*^{-/-} mice examined at more advanced embryonic stages, the olfactory bulbs appeared to be somewhat compressed along the dorsal-ventral axis (Figure 2D and Figure S2). Nonetheless, the dramatic decrease of lateral innervation remained a consistent feature in mutant olfactory bulbs,

with the olfactory sensory axons appearing to accumulate along the ventral-medial region of the olfactory bulb.

Compensation of Olfactory Bulb Innervation Defects in *Igf1* or *Igf2* Mutants

The results described above demonstrate the necessity of IGF signaling for the innervation of the lateral face of the olfactory bulb by olfactory sensory axons. We next wished to determine which of the two IGF ligands is responsible for directing the axons to the lateral olfactory bulb. Based on the lateral > medial bias in IGF1 expression observed in the olfactory bulb at E14.5, it seemed plausible that IGF1 could be required for the innervation of the lateral olfactory bulb. To test this idea, we examined the projection of olfactory neurons in bulbs from mutant mice lacking either the *Igf1* or *Igf2* gene. Olfactory axons were localized using an anti-OMP antibody in *Igf1*^{-/-}, *Igf2*^{-/-}, or heterozygous littermate controls at E18.5 (Figure 3). In both *Igf1* and *Igf2* single null mutants, the pattern of sensory axon innervation in the olfactory bulb was essentially indistinguishable from controls ($n = 5$ mice for *Igf1*^{-/-}; $n = 2$ for *Igf2*^{-/-}).

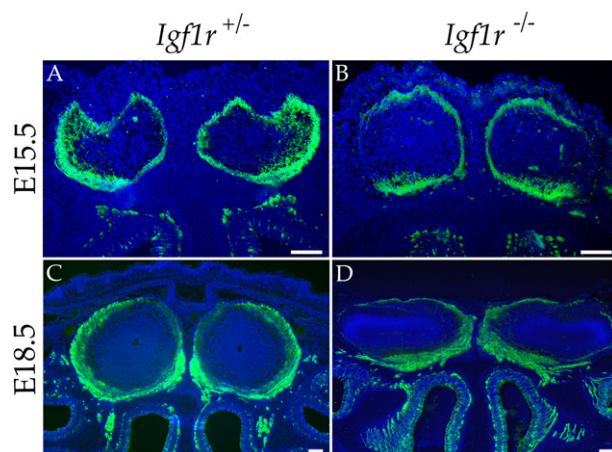


Figure 2. Innervation of the Lateral Olfactory Bulb Is Severely Reduced in the Absence of IGF Signaling

Anti- β -galactosidase staining (green) of coronal sections from heads of *OMP-IRES-tau:lacZ; Igfr^{+/-}* (A and C) and *OMP-IRES-tau:lacZ; Igfr^{-/-}* (B and D) mice reveal a deficiency in olfactory axon targeting to the lateral face of the olfactory bulb during development of the *Igfr^{-/-}* animals. (A and B) E15.5 animals show that at this early stage of olfactory map formation, the absence of IGF signaling perturbs the symmetry of ORN projections to the medial and lateral olfactory bulb (compare [A] [heterozygous control] with [B] [*Igfr* null mutant]). (C and D) A comparison at E18.5 shows a similar lack of innervation of the lateral face of the olfactory bulb in the *Igfr* null mutant (D) compared with the control (C). Nuclei were stained with Hoechst 33342 (blue). Scale bar, 100 μ m.

A straightforward and testable hypothesis explaining the difference between the *Igfr* null phenotype and the lack of mutational consequences in the absence of either IGF ligand is that each of the ligands can compensate for the lack of the other. When we analyzed the innervation of olfactory bulbs in the *Igfr^{-/-}; Igf2^{-/-}* double mutant, we obtained results consistent with this hypothesis. As shown in Figure 3E, lateral innervation of the olfactory bulbs is dramatically decreased in the *Igfr^{-/-}; Igf2^{-/-}* double mutant, to an extent similar to that of the *Igfr^{-/-}* null phenotype ($n = 2$ mice for *Igfr^{-/-}; Igf2^{-/-}*). Thus, elimination of IGF signaling—either by genetically ablating both known IGF ligands or their common receptor—leads to the redistribution of olfactory sensory axons away from the lateral olfactory bulb toward the ventral-medial face.

Absence of IGF Signaling Causes Aberrant Targeting of Olfactory Sensory Neurons

Each olfactory bulb normally exhibits mirror symmetry, such that olfactory neurons expressing a given odorant receptor typically project their axons to two glomerular targets: one on the lateral face and one on the medial face of the olfactory bulb (Mombaerts et al., 1996; Nagao et al., 2000; Ressler et al., 1994; Vassar et al., 1994). The reduction of lateral innervation in the olfactory bulbs from both *Igfr* and *Igf1*; *Igf2* null mutants suggests that IGF signaling has a role in forming the mirror symmetry inherent in the olfactory sensory map. In one hypothesis, IGF signaling guides a subset of olfactory axons to the lateral bulb. Alternatively, IGF signaling does not function in the process of axon guidance, but rather provides trophic support with differential effects on

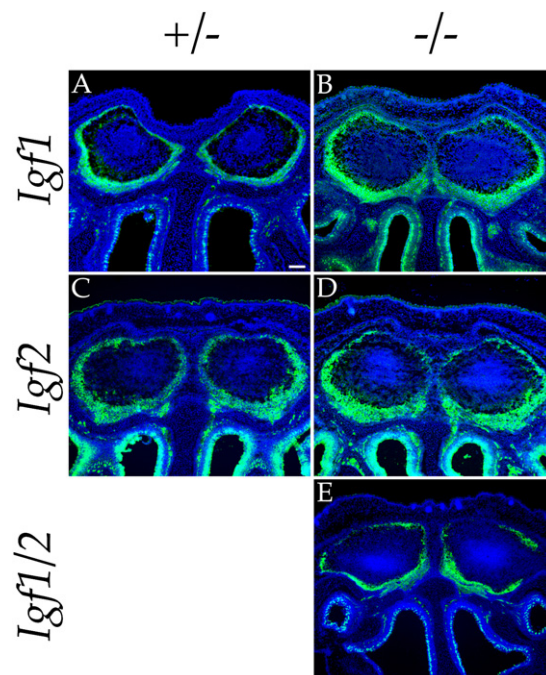


Figure 3. Effects of Targeted Mutations in the *Igf1* and *Igf2* Genes on Olfactory Bulb Innervation

Immunolocalization of OMP expression (green) in coronal sections at E18.5 reveals that *Igf1* and *Igf2* single nullizygous mice do not have significant olfactory patterning defects (compare [A] with [B] and [C] with [D]). However, the elimination of both *Igf1* and *Igf2* in the *Igfr^{-/-}; Igf2^{-/-}* mutant results in a severe decrease in innervation of the lateral olfactory bulbs, similar to the phenotype seen in the *Igfr* nullizygote (compare [E] to Figure 2D). Cell nuclei were stained with Hoechst 33342 (blue). Scale bar, 100 μ m.

subpopulations of olfactory neurons. In this latter scenario, neurons extending axons to the lateral olfactory bulb are more dependent on IGF signaling for their survival than medially directed neurons. It has been shown, for example, that IGF signaling acts as a trophic factor for some neurons in vitro (Vincent et al., 2004) and in vivo (Chrysis et al., 2001; Ye et al., 1996). Thus, the loss of sensory axon innervation in the lateral portion of the olfactory bulb could be caused by the death of laterally projecting neurons before or soon after their projections reach the bulb. We reasoned that if laterally projecting cells die prematurely in the absence of IGF signaling, an olfactory neuron population expressing a single odorant receptor would form only the medial glomerulus instead of forming the usual pair of glomeruli.

To discriminate between these two hypotheses, we examined the projection pattern of olfactory neurons expressing the same odorant receptor, receptor P2. Axons projecting from cells expressing the P2 odorant receptor were visualized immunohistochemically using mice harboring an IRES-tau:lacZ insertion at the P2 locus (Mombaerts et al., 1996). Sections of olfactory bulbs from E18.5 mice wild-type (data not shown) or heterozygous at the *Igf1* locus were stained for β -galactosidase and showed a normal projection pattern of P2 olfactory neurons; axons formed glomeruli on both the medial and lateral faces of the olfactory bulb, a few hundred microns apart along the rostral-caudal axis (Figures 4A and 4B). On the other hand, in E18.5 *Igfr^{-/-}*

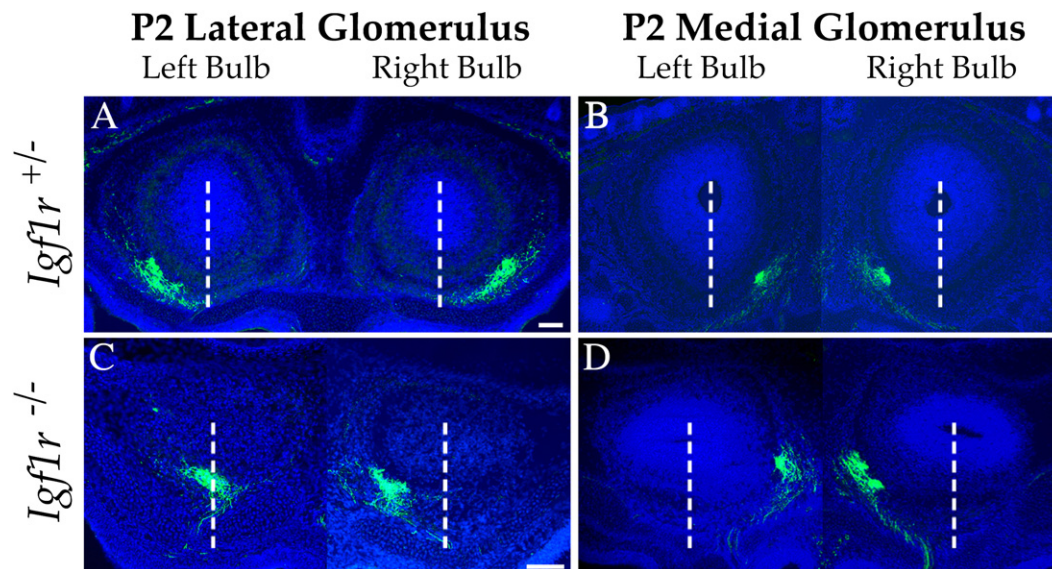


Figure 4. Misrouting of Odorant Receptor-Specific Olfactory Axons in the Absence of IGF Signaling

To localize the glomeruli of one class of odorant-receptor-specific olfactory axons, E18.5 P2-IRES-tau:lacZ mice either heterozygous (A and B) or nullizygous (C and D) at the *Igf1r* locus were stained with an anti- β -galactosidase antibody (green). (A and B) In *Igf1r*^{+/−} mice, P2 neurons project to two glomeruli per olfactory bulb, one on the lateral (A) and, more caudally, one on the medial (B) face of the bulb. The position of these glomeruli is stereotyped between animals. (C and D) P2 neurons still form two glomeruli in the *Igf1r* nullizygous background. However, the lateral glomerulus (C) is shifted toward the medial hemisphere of the bulb, resulting in a distortion of the medial-lateral mirror symmetry of olfactory bulb innervation. Dashed white lines demarcate the midlines of each bulb. Note that in this particular individual, the lateral glomerulus in the right-hand bulb has shifted well beyond the midline. The medial glomeruli in the *Igf1r* nullizygote (D) appears to be in a position similar to that observed in the control (B), with only a small dorsomedial shift, which is likely due to the compression of the map into a smaller area of the bulb's surface. In this example, the medial P2 axons form a doublet in the right bulb (D), a common phenomenon seen with this glomerulus (Royal and Key, 1999). Images of the left and right bulb are combined for clarity. Nuclei were stained with Hoechst 33342 (blue). Scale bar, 100 μ m.

littermates the axons from P2 neurons failed to extend into the lateral half of the bulb, and instead formed a glomerulus in the ventral-medial region (e.g., left bulb in Figure 4C). In some cases the position of the lateral glomerulus was shifted beyond the midline, into the medial half of the bulb (e.g., right bulb in Figure 4C). More caudal sections containing the medial P2 glomeruli revealed a slight shift in the position of these glomeruli in the *Igf1r*^{−/−} bulb to more dorsal positions (Figure 4D), which was most likely due to the compression of the total sensory axon projection onto a smaller area of the olfactory bulb.

Similar results were obtained from seven P2-IRES-tau:lacZ; *Igf1r*^{−/−} mice (versus six littermate controls). To quantitate this effect, we measured the positions of the P2 medial and P2 lateral glomeruli along the medial-lateral axis of the olfactory bulb, where in a given section containing a P2 glomerulus, 0 corresponds to the medial margin of the mitral cell layer and 1.0 corresponds to the lateral margin of the mitral cell layer (Cutforth et al., 2003); a value of 0.5 approximates the midline of the olfactory bulb in each section. Using this metric, we found that the P2 medial glomerulus position was 0.08 ± 0.08 in control (wild-type or heterozygous) mice (mean \pm SEM; $n = 13$ glomeruli), compared to -0.003 ± 0.02 in the mutant ($n = 6$); this difference is not statistically significant (two-tailed t test: $p = 0.48$). By contrast, the P2 "lateral" glomerulus on average was shifted beyond the bulb midline in the *Igf1r*^{−/−} background (position = 0.38 ± 0.09 , $n = 6$), which is significantly different from the position measured in control animals (1.05 ± 0.03 , $n = 6$; $p < 0.00004$). These

observations indicate that the olfactory axon patterning defect in *Igf1r*^{−/−} mice is due to the misrouting of axons to ventral-medial locations in the bulb, and is not due to the catastrophic loss of laterally projecting neurons.

IGF Can Serve as a Chemoattractant for Axon Growth Cones of Olfactory and Cerebellar Neurons

Our genetic evidence has revealed an unexpected role of IGF signaling in the patterning of sensory axon connections in the olfactory bulb. From these *in vivo* studies, however, it is unclear whether IGF acts as an instructive cue that directs the migration of olfactory axon growth cones, or alternatively functions indirectly to shape the olfactory sensory projection. To distinguish between these possibilities, we assessed the effects of IGF on axon growth cone migrations in cultured olfactory neurons. Olfactory neurons were isolated from neonatal rats and cultured for 24–48 hr. Cells were then transferred to serum-free medium, and actively migrating growth cones were identified and selected to test the action of IGF1 delivered from a pressure-injection pipette (Zheng et al., 1994). As shown in Figures 5A–5C, growth cones were attracted to a gradient formed by a localized source of 200 μ g/ml (26 μ M) IGF1 ($24^\circ \pm 5.1^\circ$ [mean \pm SEM], $n = 12$ growth cones scored). Under the conditions of this assay, the initial concentration of ligand at the growth cone is roughly 1/1000 the concentration in the pipette (Zheng et al., 1994), or 26 nM. This concentration corresponds to $\sim 10 \times K_d$ of IGF1 for IGF1R (Jones and Clemmons, 1995); thus, the observed

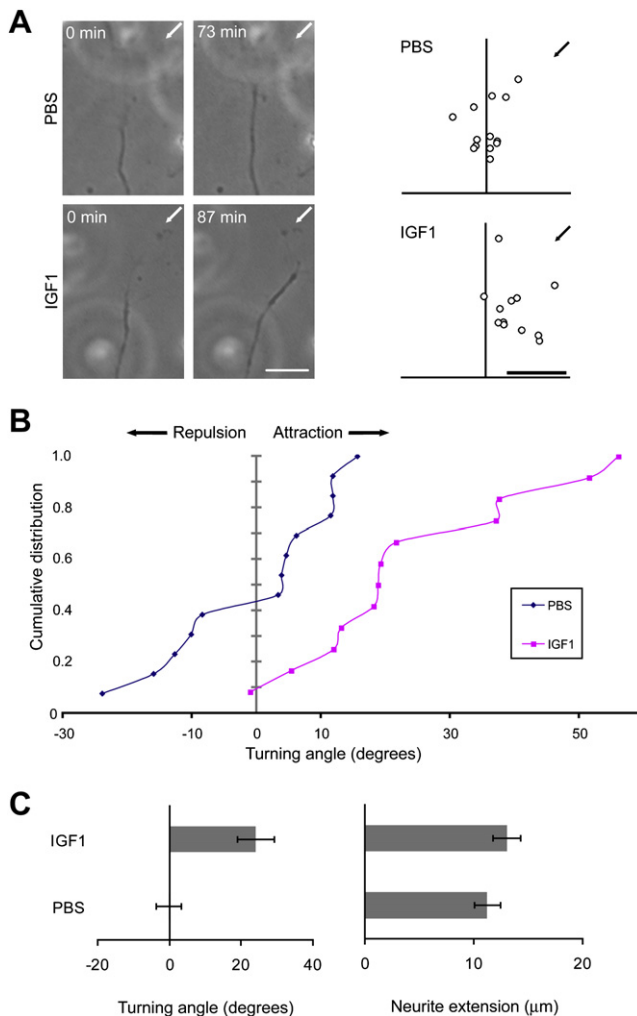


Figure 5. IGF1 Is a Chemoattractant for Growth Cones of Olfactory Receptor Neurons In Vitro

Growth cone turning assays were performed on cultured olfactory neurons isolated from neonatal rats.

(A) Images of representative olfactory receptor neuron growth cones before and after exposure to a gradient of IGF1 (200 $\mu\text{g}/\text{ml}$) or PBS solution (left-hand panels). Arrows indicate the direction of the micropipette. Scattered points in the right-hand panel depict the final positions of growth cones. Scale bar, 10 μm .

(B) Cumulative distribution of growth cone turning angles, showing attraction of growth cones to IGF1. PBS control: $n = 13$ growth cones scored; IGF1: $n = 12$.

(C) Mean turning angle (left) and neurite extension rate (right) in response to IGF1 and PBS. Responses under these two conditions were significantly different with regard to turning angle (two tailed t test: $p < 0.0001$) but not neurite extension rate ($p = 0.31$). Error bars = SEM.

effects occurred within the upper physiological range of IGF1/IGF1R action. The responses to IGF1 were significantly different from growth cone responses to a phosphate-buffered saline (PBS) control ($-0.19^\circ \pm 3.5^\circ$, $n = 13$; two tailed t test: $p < 0.001$).

It is possible that the observed attraction of olfactory neuron growth cones to IGF1 is due to increased rates of axon growth, and not the turning of growth cones per se. To address this possibility we calculated the net growth of the same axons used in

the calculations of turning angles shown in Figures 5B and 5C. We found no differences in the growth of axons exposed to PBS or IGF1 ($p = 0.31$; Figure 5C). Thus, under these assay conditions IGF1 can elicit olfactory neuron growth cone attraction independent of any effect on axon growth rate. These results indicate that IGF functions as a chemoattractant to instruct the trajectory of growing olfactory axons.

To determine whether IGF can serve more generally as a chemoattractant cue for other neuronal cell types, we examined the effects of IGF1 on cultured cerebellar granule cells. Cerebellar granule neuron cultures are a well-established system for the evaluation of the activity of secreted proteins on axon guidance and for the elucidation of the signaling mechanisms underlying growth cone responses to such cues (Li et al., 2005; Xiang et al., 2002). Moreover, the relative ease of culturing cerebellar granule neurons (as compared with olfactory neurons) provides an opportunity to dissect the intracellular events downstream of IGF signaling. To perform the assays, granule neurons were isolated from rat cerebellum at postnatal (P) day 0 and placed in culture for 12–24 hr. Cells were then transferred to IGF-free medium for 30 min prior to exposure to a gradient generated by a point source of IGF1 (Zheng et al., 1994). Similar to our observations with cultured olfactory neurons, granule neuron growth cones were attracted to a gradient formed by a localized source of 20 $\mu\text{g}/\text{ml}$ (2.6 μM) IGF1 ($9.4^\circ \pm 1.8^\circ$, $n = 42$ growth cones scored; Figures 6A–6C). The turning of growth cones in response to IGF is dose-dependent, as elevating the concentration of IGF1 to 200 $\mu\text{g}/\text{ml}$ in the source pipette increased the turning angle to $13.3^\circ \pm 4.4^\circ$ ($n = 35$; Figures 6A–6C). These responses were significantly different from growth cone responses to PBS ($-1.3^\circ \pm 1.7^\circ$, $n = 31$; two tailed t test: $p < 0.0001$ for 20 $\mu\text{g}/\text{ml}$ IGF1 versus PBS and $p < 0.01$ for 200 $\mu\text{g}/\text{ml}$ IGF1 versus PBS).

PI3 kinase is a downstream target of IGF1R (Yamamoto et al., 1992); among other actions, it appears to mediate the activity of multiple axon guidance cues (Ming et al., 1999; Song and Poo, 1999). We therefore asked whether the observed attraction of granule neuron growth cones to IGF1 is PI3 kinase-dependent, using two different approaches. First, we determined whether Akt, a direct downstream target of PI3 kinase (Yamamoto et al., 1992), is phosphorylated in response to IGF1. Serum-starved cultures of cerebellar granule neurons were treated with different concentrations of IGF1 (2, 20, or 200 ng/ml) for 30 min, and the presence of phosphorylated Akt was evaluated by western blotting using an anti-phospho-Akt antibody. As shown in Figure 6E, IGF1 elicited a dose-dependent increase in phospho-Akt, indicating that PI3 kinase is indeed activated by IGF1 in these cells. In a second experiment, we measured growth cone turning responses to IGF1 in the presence of LY294002, a specific PI3 kinase inhibitor. We observed that growth cones did not show any attraction to 200 $\mu\text{g}/\text{ml}$ IGF1 with 10 μM LY294002 present in the culture medium (Figures 6B and 6C; turning angle = $-2.3^\circ \pm 2.4^\circ$, $n = 26$; $p > 0.7$ compared with PBS control), demonstrating that the attraction of growth cones to IGF1 is dependent on PI3 kinase signaling. Finally, we found no differences in the growth of axons exposed to PBS, low or high levels of IGF1, or IGF1 in the presence of LY294002 (Figure 6D). Thus, under these assay conditions IGF1 can elicit growth cone attraction independent of any effect on axon growth rate. Together these results indicate

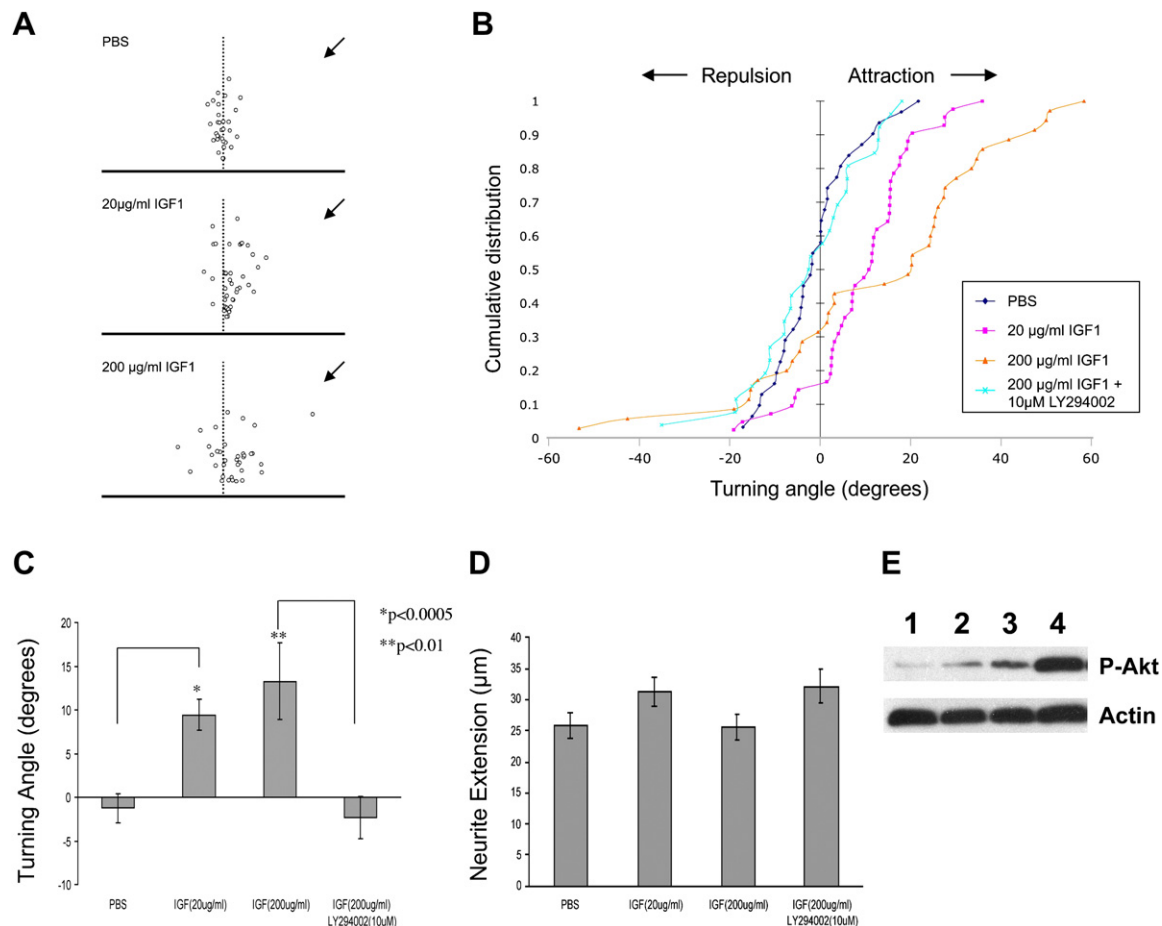


Figure 6. IGF1 Is a Chemoattractant for Cerebellar Granule Neuron Growth Cones In Vitro

Growth cone turning assays were performed on cultured rat cerebellar granule neurons. Actively migrating growth cones were exposed to a gradient of IGF1 from a point source set at an initial 45° angle.

(A) Scatter plots showing the relative angle (x axis) and total neurite extension (y axis) of individual growth cones following exposure to PBS, 20 µg/ml IGF1, or 200 µg/ml IGF1, as indicated. For each plot, the origin corresponds to the center of the growth cone at the beginning of the experiment, and the direction of the gradient is indicated with an arrow.

(B) Cumulative distribution of turning angles, showing that growth cones are attracted to a gradient of IGF1 in a dose-dependent manner when compared with PBS control, and that this attraction is abolished in the presence of the PI3 kinase inhibitor LY294002. PBS control: n = 31 growth cones assayed; 20 µg/ml IGF1: n = 42; 200 µg/ml IGF1: n = 35; 200 µg/ml IGF1 + 10 µM LY294002: n = 26.

(C) Quantitation of the data shown in (A) demonstrates that the attraction of growth cones to IGF1 is statistically significantly different as compared with control (PBS; see text for details). Inhibition of PI3 kinase with LY294002 results in a significant reduction in IGF1-mediated attraction. Error bars = SEM.

(D) Measurement of net extension of the same growth cones over a 30 min period shows no effect of IGF1 on neurite outgrowth under these assay conditions. Error bars = SEM.

(E) Western blotting for phospho-Akt, a downstream target of IGF1R/PI3 kinase, reveals a dose-dependent accumulation of phosphorylated Akt in response to IGF1 in cerebellar granule cells (top panel). An anti-actin blot from the same gel serves as a loading control (bottom panel). Lane 1, no IGF1; lane 2, 2 ng/ml IGF1; lane 3, 20 ng/ml IGF1; lane 4, 200 ng/ml IGF1.

that IGF can serve as a chemoattractant cue for growth cones of both olfactory neurons and cerebellar granule neurons, suggesting a general role of IGF signaling in axon guidance.

DISCUSSION

IGF Signaling Guides the Formation of the Olfactory Sensory Map

In the present study, we identify IGF signaling as an important determinant in the development of the olfactory sensory map.

Specifically, IGF signaling is required for the correct positioning of glomeruli in the lateral olfactory bulb. In IGF mutants, glomeruli normally positioned in the lateral half of the bulb are shifted to ventral-medial locations. The abnormal accumulation of fibers in the ventromedial bulb suggests a rotation and compression of the sensory map from dorsal-lateral positions toward the ventral midline. We interpret these observations to suggest that IGF-mediated signaling in the ingrowing olfactory axons is required for their proper innervation in the olfactory bulb. This view should be tempered by the possibility that the olfactory phenotype of

IGF mutants is due instead to indirect effects that lead secondarily to axonal misrouting. For example, loss of IGF signaling could cause a change in gene expression within the olfactory neurons, causing them to respond differently to target-derived axon guidance cues. Alternatively, the misplacement or absence of cells in specific bulb regions could result in the absence of innervation where such cells are missing. It should be noted, however, that the convergence of olfactory sensory axons to both medial and lateral sites can occur in mouse mutants in which olfactory bulb neurons are either missing or disorganized (Bulfone et al., 1998; Royal et al., 2002; St John et al., 2003). Moreover, we found that IGF1 can serve as a chemoattractant for growth cones of cultured olfactory neurons, consistent with the notion that IGF is acting directly on olfactory axons in vivo.

IGF Signaling and Axon Guidance

It is generally thought that the IGFs function predominantly as growth-promoting factors (Efstratiadis, 1998). Although a loss of cells in specific brain nuclei and axons of certain nerve tracts has been observed in an *Igf1*^{-/-} mutant (Beck et al., 1995), there has been no clear evidence to suggest effects on neuronal patterning that are not secondary to perturbations in cell proliferation or cell survival. Nonetheless, a recent study has demonstrated that the axons forming the corticospinal tract are dependent on IGF signaling for growth; IGF1 applied to neurons in culture stimulated the rate of axon extension, and inhibition of IGF1R with a function-blocking antibody in vivo perturbed axon outgrowth (Ozdinler and Macklis, 2006). Here we extend these findings by showing an unexpected role for IGF signaling in axon guidance. In the olfactory system, disruption of IGF signaling causes a dramatic misrouting of axons normally destined to innervate the lateral olfactory bulb. We further show that IGF functions as a chemoattractant for growth cones of cultured olfactory neurons and cerebellar granule neurons, independent of any effect IGF may have on promoting axon outgrowth. We hypothesize that IGFs have the potential to serve more broadly as instructive cues that guide the migrations of growing axons in the developing nervous system. Indeed, PI3 kinase, a downstream effector of IGF signaling through IGF1R (Yamamoto et al., 1992), has been shown to mediate growth cone turning in response to a variety of axon guidance cues (Song and Poo, 1999).

From our present studies it is not entirely clear whether olfactory axons projecting to the lateral olfactory bulb depend on IGF signaling for axon outgrowth, as in the corticospinal tract (Ozdinler and Macklis, 2006). However, we never observe axons stalled outside of the olfactory bulb in IGF mutants (e.g., see Figure 2), and a close examination of P2 axon trajectories indicates that axon outgrowth is not inhibited in IGF mutants. In wild-type mice, P2 axons entering the bulb through openings in the lateral cribriform plate turn laterally and extend toward their target glomerulus (Figure 7C). In contrast, P2 axons crossing the lateral cribriform plate appear to turn medially upon entering the bulb in the *Igf1*^{-/-} mutant, and extend across the bulb's ventral surface to form a misplaced glomerulus (Figure 7D). In both cases the labeled axons traverse a comparable distance after entering the olfactory nerve layer, suggesting that IGF signaling is not strictly required for the outgrowth of olfactory axons.

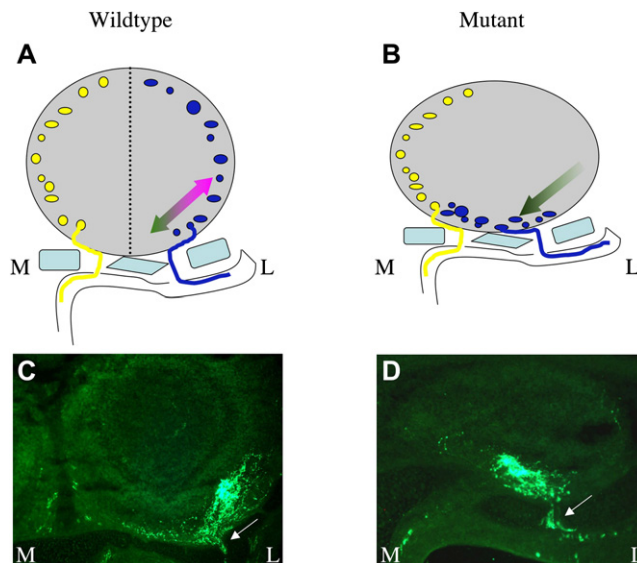


Figure 7. Model of IGF Activity in the Formation of the Olfactory Sensory Map

(A) Under normal conditions, medially (gold) and laterally (blue) disposed olfactory axons cross the cribriform plate (light blue) and extend to medial and lateral glomeruli, respectively, forming a mirror symmetry in the olfactory bulb (dotted line represents the plane of symmetry, which extends out of the plane of the figure). We hypothesize that the position of a lateral glomerulus is determined through a "push-pull" mechanism. In this model, IGF ligands expressed in the olfactory bulb attract axons exiting the lateral cribriform plate to grow dorsolaterally (magenta arrow). This dorsolateral attraction is opposed by an unknown cue or cues that influence the axons to grow ventral-medially (dark green arrow). Together these opposing forces play a role in determining the positions of glomeruli in the lateral olfactory bulb.

(B) In IGF mutants, axons no longer respond to IGF (*Igf1*^{-/-}) or IGFs are missing (*Igf1*^{-/-}; *Igf2*^{-/-}), leaving the medial "push" (dark green arrow) unopposed by the IGF-dependent lateral "pull." This results in a severe distortion of the mirror symmetry within the olfactory bulb, with a lateral → ventral-medial rotation and compression of the sensory map. Axons that normally extend to the medial olfactory bulb are largely unaffected by the absence of IGF signaling, and are probably responding to another attractive cue or cues to enter the medial bulb.

(C and D) Examples of tau:lacZ-labeled P2 axons from the lateral olfactory epithelium as they penetrate and cross the cribriform plate (arrows) and extend to their target glomeruli. In the wild-type background, axons turn and extend laterally upon exiting the cribriform plate (C). In the *Igf1*^{-/-} mutant, axons instead turn medially and extend a comparable distance to an ectopic site in the ventromedial bulb (D). Thus, the observed distortion of the olfactory sensory map in IGF mutants is likely the result of the misrouting of axons normally destined to innervate the lateral olfactory bulb.

Mechanisms of Olfactory Sensory Map Formation

The stereotypic nature of the olfactory sensory map suggests that the precise convergence and targeting of sensory axons in the olfactory bulb is the result of a response of specific sensory axons to spatially restricted guidance cues in the target tissue and along the axonal trajectory. In one model, a hierarchy of such guidance cues directs axons of neurons expressing the same odorant receptor to their common glomerular targets in the olfactory bulb (Lin and Ngai, 1999; St John et al., 2002). For example, neurons expressing a specific odorant receptor are segregated within defined zones in the epithelium along

the dorsomedial-ventrolateral axis (Miyamichi et al., 2005; Ressler et al., 1993; Vassar et al., 1993). While axons arising from each zone project to a corresponding dorsal-ventral zone in the olfactory bulb (Miyamichi et al., 2005; Mori et al., 1999), within these latter zones odorant receptor-specific axons converge to form discrete glomeruli. Zone-to-zone projection may be established by a mechanism that restricts the olfactory axons to broad domains in the bulb (Cho et al., 2007), while additional levels of control direct axons to converge to their appropriate glomeruli within these domains. Interestingly, the odorant receptor itself—via cAMP signaling—influences glomerular position along the anterior-posterior axis of the olfactory bulb (Imai et al., 2006; Mombaerts et al., 1996; Wang et al., 1998), although it is not the sole determinant of the final position of the glomerulus. Thus, zone-to-zone projections restrict axons along the dorsal-ventral axis of the olfactory bulb and odorant receptors influence glomerular positioning along the anterior-posterior axis. Here we show that IGF signaling shapes the olfactory axon projection along the medial-lateral axis, the bulb's third principal axis. The sorting of axons to the medial and lateral hemispheres of the olfactory bulb represents a key developmental choice necessary for the establishment of mirror symmetry in the olfactory sensory map.

What mechanisms are used to influence this choice? Axons innervating the medial or lateral olfactory bulb originate from neurons found in medial or lateral positions of the olfactory epithelium, respectively (Levai et al., 2003). In a simple scenario, axons would merely need to grow “up” into the bulb in order to innervate the medial and lateral hemispheres. Consistent with this idea, the chemorepellant *Sema3A* is expressed in the medial bulb and has been suggested to provide a midline barrier for olfactory axons expressing neuropilin-1, the *Sema3A* receptor (Schwartz et al., 2000; Taniguchi et al., 2003). However, a null mutation in the *Sema3A* gene results in only a slight anterior-medial shift of the two mirror-symmetric maps (Taniguchi et al., 2003). The lateral > medial gradient of IGF1 in the rostral olfactory bulb at E14.5 (Figure 1) suggests that the differential localization of IGF1 may attract some olfactory axons preferentially to the lateral bulb. However, this gradient is subtle, is restricted to the rostral olfactory bulb, and disappears by E15.5 (data not shown). It is also possible that any one of the numerous IGF binding proteins encoded in the genome (Efstratiadis, 1998) may sculpt the spatial distribution of available IGF ligands along the olfactory projection. Yet a null mutation in the *Igf1* gene does not cause a demonstrable perturbation in the olfactory sensory projection (Figure 3), suggesting that the mechanisms influencing medial versus lateral projection decisions likely involve a complex interplay between multiple factors.

While it is formally possible that the response of olfactory neurons to other axon guidance cues is itself dependent on IGF signaling, we favor a model in which olfactory axons are guided by IGFs (and possibly other factors) to their final glomerular position (Figure 7). In this model, all olfactory axons, regardless of their origin, are attracted to the medial bulb. Axons originating from the lateral olfactory epithelium therefore require a counterbalancing lateral attraction in order to extend into the lateral hemisphere of the olfactory bulb. We propose that IGF ligands serve as this attractive cue, with the ultimate location of a given glomerulus

in the lateral hemisphere being a function of the medial “push” and lateral “pull” imposed on the sensory axon growth cones (Figure 7A). In the absence of IGF signaling—as in the IGF mutants studied here—the lateral pull is severely reduced, and the medial push (which may arise from an attractive medial cue or repulsive lateral cue) is revealed, resulting in the misrouting of axons to ectopic locations in the ventral bulb (Figure 7B). Although our model invokes the activity of a hypothetical factor to provide a medial push to the axons, an analogous “push-pull” mechanism has been described in the development of the retinotopic map, where countergradients specify the location of retinal ganglion cell axon terminals in the target tissue (Schmitt et al., 2006).

Why do the lateral axons not target to the same location as the medial glomerulus in IGF mutants? Without the appropriate attractive cue to turn laterally, the lateral axons find themselves in a novel environment. We speculate that they form glomeruli in positions based upon the molecular cues present in their new target environment, which may be very different from that found in the lateral hemisphere. It is interesting to note that the position of the medial glomerulus is largely unaffected in *Igf1*^{−/−} mice; axons innervating this glomerulus appear to follow their normal targeting strategy independent of IGF signaling. The behavior of these axons may reflect the presence of other attractive cues in the medial bulb that compensate for the absence of IGF signaling in the mutant background.

It remains unclear at present what factors or cues specify the exact location of individual glomeruli. Nonetheless, the data presented here clearly identify a role of IGF signaling in the formation of the olfactory sensory map and add the IGFs to a growing list of molecules involved in the patterning of neuronal connections in the developing nervous system.

EXPERIMENTAL PROCEDURES

Mouse Strains

All mouse strains used in this study were on a mixed 129/B16 background. *Igf1* and *Igf2* mutants are described in DeChiara et al. (1990) and Liu et al. (1993). *Igf1r* mutants, described in Liu et al. (1993), were crossed with *OMP-IRES-tau:lacZ* and *P2-IRES-tau:lacZ* mice described in Mombaerts et al. (1996).

Immunohistochemistry

Whole heads from different embryonic stages were fixed for 2 hr at room temperature in 4% paraformaldehyde, followed by an overnight incubation in 30% sucrose, 1X PBS at 4°C. Tissue was then mounted in TBS tissue freezing medium (Triangle Biosciences) and frozen at −80°C until sectioned. Cryosections were taken at 12 μm thickness unless indicated otherwise.

For immunohistochemistry, slides were incubated in PBST (1X PBS, 0.1% Triton X-100) for 5 min followed by a 1 hr incubation in blocking solution (1X PBS, 10% heat-inactivated normal goat serum [HINGS], and 0.1% Triton X-100) before primary antibody was added. Rabbit anti-β-galactosidase (1:900, Cappel) or rabbit anti-GFP (purified IgG; 1:900, Invitrogen) were incubated for 1 hr at room temperature followed by incubation at 4°C overnight. For IGF1R staining, sections were incubated in 3% H₂O₂ for 45 min prior to incubation in blocking solution; chicken anti-IGF1R (1:10, Upstate) was incubated for 2 hr at room temperature followed by incubation at 4°C overnight and visualized using an HRP-conjugated goat anti-chicken antibody (1:200, AbCam) and Cy3 tyramide (Perkin Elmer). IGF1R was also localized using a rabbit anti-IGF1R antibody (1:50, Cell Signaling Technology) following treatment of tissue sections in boiling sodium citrate buffer. Goat anti-IGF1 and goat anti-IGF2 (R&D systems) were each used at 1:20 dilution. Goat anti-OMP (Wako) was used at

1:1000. For goat primary antibodies, donkey serum replaced goat serum in the blocking solution. Signal was detected using Alexa 488-, Alexa 568-, or Alexa 594-conjugated secondary antibodies (Invitrogen). Sections were counterstained with Hoechst 33342 to visualize the positions of nuclei. RNA in situ hybridizations were performed as described previously (Specia et al., 1999).

Neuronal Cell Culture and Growth Cone Turning Assays

Olfactory septa and turbinates were collected from P0–P2 neonatal rats and incubated in papain (20 units/ml in PBS) at 37°C for 30 min. The solution was then removed and replaced with 10% fetal bovine serum (FBS) in Neurobasal medium, after which the remaining bony pieces and large aggregates were triturated gently with a glass pipette. The dissociated cells were pelleted by centrifugation, resuspended in prewarmed olfactory neuron culture media (Neurobasal plus 2% B27, 10 µg/ml insulin, 2 mM Glutamax, 10 ng/ml BDNF, and 25 ng/ml NGF) and plated on laminin-coated coverslips. After culturing for 24–48 hr at 37°C, coverslips were transferred to a heated chamber on the stage of a microscope and incubated in serum-free L15 medium for at least 30 min at ~37°C before the growth cone turning assay. Cerebellar granule neurons from P0 rats were dissociated and plated at low density (10⁵/dish) onto laminin-coated coverslips (Xiang et al., 2002). After culturing for 12–24 hr at 37°C, coverslips were transferred to a heated chamber on the stage of a microscope and incubated in serum-free L15 medium for at least 30 min at ~37°C.

Microscopic gradients of IGF were produced as described previously (Zheng et al., 1994), except that the tip of the pipette was located 75 µm away from the growth cone center. Images of neurites were recorded and analyzed using Scion Image 4.0.2. Turning angle was defined as the angle between the original direction of the neurite's extension and a straight line connecting the growth cone position at the start and the end of the assay period. Procedures for both cell types were identical, except that the final positions of the olfactory neuron growth cones were measured following 60 to 90 min exposure to the gradient (versus 1 hr for cerebellar granule neurons), and only neurites that extended >5 µm during the 60 to 90 min period were included in the analysis for olfactory neurons. For pharmacological treatment of cerebellar granule neurons, LY294002 was applied 30 min before the turning assay and maintained throughout the duration of the assay.

Western Blotting

Cerebellar granule cells were cultured at high density (2 × 10⁵/dish) in 35 mm tissue culture dishes and were serum starved for 2 hr before stimulation with IGF1. Cells were harvested in lysis buffer (0.1% SDS, 1% Nonidet P-40, 1% glycerol, 50 mM HEPES [pH 7.4], 2 mM EDTA, and 100 mM NaCl; 200 µl/dish). Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred onto PVDF membranes. Blots were blocked for 3 hr at room temperature in 5% BSA, incubated with a polyclonal antibody specific for phospho-Akt (1:1000, R&D Systems) overnight at 4°C, rinsed, and incubated for 1 hr at room temperature with an HRP-conjugated goat anti-rabbit IgG (1:10,000; Biorad). Chemiluminescence detection was performed with an ECL kit (Pierce).

SUPPLEMENTAL DATA

The Supplemental Data for this article can be found online at <http://www.neuron.org/cgi/content/full/57/6/847/DC1/>.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute on Deafness and Other Communications Disorders (J.N.), the National Science Foundation of China (X.-b.Y., 30625023), and the National Cancer Institute (A.E., 1P01 CA97403, Project 2), and a gift from the Berrie Foundation (A.E.). We thank Richard Axel for generously providing mouse strains and Mu-ming Poo for insightful discussions throughout the course of this study.

Received: April 11, 2007

Revised: September 25, 2007

Accepted: January 14, 2008

Published: March 26, 2008

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